The University of Chicago

FOUNDED BY JOHN D. ROCKEFELLER

THE MECHANISM OF NATURAL AND ACQUIRED STREPTOCOCCUS IMMUNITY

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE

OGDEN GRADUATE SCHOOL OF SCIENCE

IN CANDIDACY FOR THE

DEGREE OF

DOCTOR OF PHILOSOPHY

(DEPARTMENT OF PATHOLOGY AND BACTERIOLOGY)

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(From the Memorial Institute for Infectious Diseases, Chicago.)

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INTRODUCTION.

A great deal of work has been done on the mechanism of streptococcus immunity both with normal and with immunized animals, but many points are still left entirely in the dark. In the experiments described in this paper, an attempt is made to throw additional light on some of these points, but on account of the many inherent difficulties, the results which I have achieved by no means exhaust the problem.

Shortly after the publication of his classical paper on phagocytosis in daphnia, Metchnikoff* made a study of phagocytosis of streptococci in the human body, in cases of erysipelas. He concluded from his observations that streptococci gain entrance through abrasions of the skin, multiply and set up an inflammation. At the same time there is a gathering of microcytes about the streptococci, and these take up the latter and destroy them. Macrocytes are also found in considerable numbers, but they do not take up the streptococci, but have, nevertheless, a phagocytic action in that they take up and remove dead and disabled microcytes.

In 1895 Denys and Leclef* studied the mechanism of immunity in rabbits which had been repeatedly injected with small but gradually increased doses of virulent streptococci. Their conclusions may be briefly stated as follows: Immune rabbit serum is not so good a culture medium for streptococci as normal serum but it does not possess any marked streptococcidal powers. The cell-free fluid from a leucocytic exudate

*Virchow's Archiv. 1887, cvii., 209.
**La Cellule, 1895, XI, 177.

retards multiplication of virulent streptococci and sometimes kills them. Normal leucocytes in normal serum, and leucocytes from an immune rabbit suspended in normal serum, do not greatly retard multiplication of virulent streptococci. Leucocytes from a normal rabbit, or those from an immune rabbit, suspended in immune rabbit serum rapidly take up and destroy virulent streptococci. The serum has acquired something in the process of immunization which neutralized something in the cocci by virtue of which they were protected against phagocytosis.

Denys and Marchand* showed that there is better phagocytosis of virulent streptococci when inoculated into a suspension of rabbit's leucocytes in normal rabbit serum to which had been added 1 per cent. of immune horse serum, than when they were inoculated into the same mixture of leucocytes and rabbit serum with the addition of 1 per cent. of normal horse serum.

Bordet* was able to find no protection against streptococci in normal and immunized rabbits and guinea-pigs except that due to phagocytosis. If a rabbit was treated with antistreptococcic serum, and later injected with several times the minimum fatal dose of streptococci, the organisms were all taken up by phogacytes and destroyed. In untreated rabbits he also found phagocytosis, but the organisms soon got the upper hand and the rabbit died of streptococcus infection.

Marchand* studied phagocytosis of virulent and non-virulent streptococci, and came to the conclusion that the failure on the part of the leucocytes to take up virulent streptococci depends on a physical property of the organisms and not on a secretion. Tchistovitch** injected rabbits intravenously with fatal doses of a highly virulent streptococcus, killed the animals in one-fourth to six hours and examined the organs for evidence of phagocytosis. He was always able to find some phagocytosis in the lungs and in the livers of these animals, and concluded that this phagocytosis of virulent streptococci may be due to the fact that every culture contained some cocci which are less resistant than others, and that those are the only ones which are taken up by the phagocytes.

Simon* found very little evidence of phagocytosis, in vitro, when rabbit's leucocytic exudate was inoculated with a non-virulent streptococcus. The washed leucocytes suspended in salt solution, and also the cell-free exudate, killed non-virulent streptococci, but not the virulent organisms. Leucocytic exudate mixed with rabbit serum had no effect

*Quoted from Denys, Centralbl. f. Bact., 1898, XXIV, 685.
**Ann. de l'Inst. Pasteur, 1896, X, 104, and 1897, XI, 177.
*Archiv. de Méd. Exper., 1898, X, 253.
**An. de l'Inst. Pasteur, 1900, XIV, 802.
*Centralbl. f. Bakt. 1901, XXIX, 81 and 113.

on non-virulent streptococci. He was unable to confirm the view advanced by Bordet and others that a rabbit which is injected with streptococci into a pleural cavity containing leucocytic exudate can stand a larger dose than a rabbit of the same size which is injected in the normal pleura. The pleural exudate was produced by the injection of aleuronat suspension forty-eight hours previously. These results are quite contradictory to those of other investigators, and it was, therefore, thought desirable to repeat these experiments with leucocytes.

Neufeld and Rimpau* have shown that the leucocytes play an important role in combating streptococcus infections in immunized rabbits. They have shown also that the addition of antistreptococcus serum to a suspension of leucocytes and streptococci aids phagocytosis and that this is not due to a stimulation of the phagocytes but to an effect of the serum on the cocci. The specific substance in the immune serum they found to be fairly resistant to heat, it being unchanged by heating at 59° C. for ½ hour.

TEST TUBE EXPERIMENTS WITH LEUCOCYTIC EXUDATE FROM NORMAL ANIMALS.

The leucocytic exudate used in these experiments was obtained by injecting a 6-8% suspension of aleuronat in Na Cl solution into the right pleural cavity of a rabbit or large guinea-pig and bleeding the animal to death eight to ten hours later. The aleuronat suspension is best prepared by sterilizing the aleuronat in dry heat at 150° to 160° C. and suspending it in sterile physiological salt solution. No sodium oxalate was added to the exudate, as this salt is strongly streptococcidal, but the coagulation of the exudate was prevented by occasional agitation of the tubes during the first hour. The contents of each tube were 2 c. c., which were inoculated with three loopfuls of a twenty-four-hour broth streptococcus culture and three loopfuls were plated at intervals. Tube 7 differs from Tube 6, in that it contains the washed leucocytes which had been centrifugated out of 2 c. c. of the exudate, suspended in rabbit serum, whereas Tube 6 contained a mixture of 1 c. c. of the entire exudate + 1 c. c. of serum. The fluid of the exudate in Tube 7 was removed to show that the effect of these mixtures is not due entirely to the liquid portion of the exudate, but in a large measure to the leucocytes. The Tables show that non-virulent streptococci are destroyed by the leucocytic exudate, by mixtures of leucocytes and serum and by leucocytes in defibrinated blood. Defibrinated blood and serum alone, the heated exu-

**D. Med. Woch., 1904, XXX, 1458.

date and a suspension of leucocytes in salt solution or in heated serum have little or no effect on these organisms. The virulent streptococcus is not destroyed by the leucocytic exudate nor by a suspension of leucocytes in serum or blood. Smears made in one to two hours from tubes in which there is destruction of cocci show evidence of phagocytosis. Table 2 shows the same experiment as that shown in Table 1; except that guinea-pig's leucocytes and blood were used. With these leucocytes the

TABLE 1.

SHOWING THE EFFECT OF RABBIT'S LEUCOCYTIC EXUDATE AND MIXTURES OF EXUDATE AND BODY FLUIDS ON STREPTOCOCCI.

Strepto-cocci.		Immediately.	3 hrs.	18 hrs.
300	Leucocytic exudate	4600	0	10000
300	Exudate 58° for half hour	3250	8000	Many
300	Defibrinated blood	4800	4700	Many
300	Blood 1 c. c. + exudate 1 c. c	4000	750	1500
300	Rabbit serum	4200	4200	Many
300	Serum 1 c. c. + exudate 1 c. c.	5200	1100	Many
300	Leucocytes + serum	5000	800	Many
300	Leucocytes in heated serum	5500	3560	Many
300	Leucocytes in NaCl sol	4900	5000	Many
300	Leucocytes in NaCl sol. + Sensitized streptococci	570	32	3000
324	Leucocyte exudate	420	11	Many
324	Defibrinated blood	430	7000	Many
324	Blood 1 c. c. + exudate 1 c. c.	750	540	Many
324	Rabbit serum	552	6500	14000
324	Serum 1 c. c. + exudate 1 c. c.	320	1	Many
B104	Leucocytic exudate	3500	3600	Many
270	Leucocytic exudate	1700	2	Many

Sources of streptococci: Nos. 300 and 324 from the heart's blood of scarlet fever patients, postmortem. No. B104 from an abscess in a guinea-pig which had been injected with a fungus. This organism has been passed through 78 rabbits and is very virulent for both rabbits and guinea-pigs.

eighteen-hour plates are often sterile, or nearly so, while it is only occasionally that they are sterile when rabbit's leucocytes are used.

Special attention must be called to the fact that the suspension of leucocytes in salt solution destroys many of the *sensitized* avirulent streptococci. These results are therefore in harmony with the work of Wright and Douglas,* who have shown that phagocytosis takes place only after the bacteria have been sensitized, that is, have been acted on by the opsonin of the serum. There is no phagocytosis in a suspension of washed leucocytes in salt solution or in heated serum, when untreated

*Proc. of Royal Soc., 1903, LXXII, 357, and 1904, LXXIII, 128.

bacteria are added, but when sensitized bacteria are added to such a suspension of leucocytes there is good phagocytosis. This work was confirmed and extended by Hektoen & Ruediger** and by Bulloch & Atkin.*** In consideration of these facts we would expect that the

TABLE 2.

SHOWING THE EFFECT OF GUINEA-PIG'S LEUCOCYTIC EXUDATE AND MIXTURE OF EXUDATE AND BODY FLUIDS ON STREPTOCOCCI.

Streptococci.		Immediately.	3 hrs.	18 hrs.
300	Leucocytic exudate	6500	70	170
A	Leucocytic exudate	650	600	Many
B104	Leucocytic exudate	8000	10000	Many
300	Cell-free exudate	5500	800	6000
B104	Cell-free exudate	8000	10000	Many
300	Defibrinated blood	6000	5000	Many
300	Defibrinated blood + leucocytes	6800	240	72
300	Serum	6000	6000	Many
300	Serum + leucocytes	6000	2000	Many

Streptococcus No. 300 is not virulent for guinea-pigs. Nos. B104 and A are virulent. No. A was isolated from a peritonsillar abscess following an attack of follicular tonsillitis and was used in this experiment in the first generation on artificial media.

TABLE 3.

PHAGOCYTOSIS OF STREPTOCOCCI BY RABBIT LEUCOCYTES AND GUINEA-PIG LEUCOCYTES.

Streptococci.		Phagocytosis.
300	Rabbit leucocytes in blood	20
B104	Rabbit leucocytes in blood	0
300	Guinea-pig leucocytes in blood	30
B104	Guinea-pig leucocytes in blood	1
300	Washed rabbit leucocytes in NaCl solution	1
300	Washed rabbit leucocytes in heated rabbit serum	1.5
300	Washed rabbit leucocytes + sensitized streptococci	12

Each tube contained 2 c. c. of suspension of leucocytes + 2 c. c. of suspension of streptococci. Tubes were incubated 1 hour, smears were made and stained and the degree of phagocytosis determined by counts.

extent of destruction of streptococci in the test tubes should run parallel with the degree of phagocytosis of these organisms and Table 3 shows that this is the case.

If we read the three tables together we notice that the virulent

**Jour. of Infect. Dis., 1905, II, 128.
***Proc. of. Royal Soc., 1905, LXXIV, 379.

organism (B104) is not taken up by the rabbit leucocytes and guinea-pig leucocytes in blood, neither is this organism destroyed or killed by the leucocytic exudate and suspensions of leucocytes from these animals in blood or fresh serum. The avirulent organism (300) is ingested by the leucocytes in blood and in serum but not by washed leucocytes in Na Cl solution or in heated serum unless the cocci have been treated beforehand with fresh unheated serum (sensitized). Similarly this organism is destroyed by suspensions of leucocytes in blood or serum but not by suspension of washed leucocytes in NaCl solution or heated serum. The washed leucocytes do however destroy the *sensitized* avirulent organisms.

EXPERIMENTS WITH ORGAN CELLS.

It seems quite reasonable to suppose that the organ cells might play an important part in the protection of the body against invasion by streptococci; and with this point in mind the following experiment was carried out:

Experiment.—Two guinea-pigs, A and B, were bled to death and the blood collected under aseptic precautions. The organs were now carefully removed from the body, placed in sterile glass dishes and rubbed up: (A) with guinea-pig's serum, and (B) with defibrinated guinea-pig's blood. The organs and blood were not allowed to become cold.

TABLE 4.

SHOWING THE EFFECT OF THE SUSPENSIONS OF ORGAN CELLS OF GUINEA-PIGS ON STREPTOCOCCI.

Streptococci.		Immediately.	3 hrs.	18 hrs.
B104 +	Defibrinated blood	1800	1600	1500
B104	Blood + muscle	2000	8000	Many
B104	Blood + spleen	1900	Many	Many
B104	Blood + liver	1800	Many	Many
B104	Blood + kidney	1900	Many	Many
B104	Blood + adrenal	1500	Many	Many
B104	Blood + lymph gland	1500	6000	Many
300	Blood + bone marrow	2500	1000	Many
300	Serum	3800	3750	6000
300	Serum + leucocytes	2700	1800	1500
300	Serum + spleen	2800	5000	Many
300	Serum + liver	2000	12000	Many
300	Serum + kidney	2400	12000	Many
	Control liver + serum	0	3	9
	Control spleen + serum	0	0	0

The strain of streptococcus B104 used in this experiment has not been passed through rabbits and has lost its virulence.

The suspensions of organ cells were now introduced into small test tubes, inoculated with two loopfuls of a 24-hour broth culture of a non-virulent streptococcus, and two loopfuls were plated at intervals. The colonies that developed in each plate were carefully estimated and the results are shown in Table 4. In no instance did the suspension of organ cells have an inhibitive effect on the multiplication of the streptococci, but on the contrary the organisms multiplied more rapidly in these suspensions than in either the serum or the defibrinated blood alone. If, however, bone marrow was added to the defibrinated blood or serum, the number of streptococci in these tubes was greatly diminished during the first three to five hours.

These results agree with those found by Bail and Pettersson* in their study of the mechanism of anthrax immunity in chickens, and show that suspensions of organ cells from the guinea-pig possess no streptococcidal power, except the suspensions of bone marrow. Suspensions of marrow cells in defibrinated blood or serum kill large numbers of avirulent streptococci, but that is not the case with suspensions in salt solution. These results have also been obtained with suspensions of rabbit's bone marrow.

PHAGOCYTOSIS OF LIVING STREPTOCOCCI IN VIVO.

It having been found that leucocytes ingest and destroy streptococci in test tube experiments, the following experiment was made to show that there is phagocytosis of living streptococci in the animal body:

A guinea-pig was injected in the right pleura with 7 c. c. of aleuronat suspension. Twelve hours later he was injected in this pleura with 2 c. c. of an 18-hour glucose broth culture of a non-virulent streptococcus, and two hours after this injection he was bled to death. Several cubic centimeters of leucocytic exudate were now taken from the right pleura, under aseptic precautions. Smears made with this exudate contained no free streptococci, but many of the leucocytes contained cocci in large numbers.

Figure 1 is a photomicrograph of one of these smears showing streptococci in the body of a leucocyte.

Preparation of Slides.—By means of a sterile, soft cotton swab a small quantity of the exudate was now gently smeared on each of eight agar-covered slides; the slides were incubated in a moist chamber for four to six hours, stained and examined under the microscope. The preparation of these slides requires the following special technic. Three ordinary clean glass slides and a heavy watch glass are placed in a large

*Centralbl. f. Bakt., 1903, XXXV, 102.

Petri dish and sterilized in dry heat. When the Petri dish has cooled sufficiently to be handled the slides are taken out with sterile forceps and hot glucose agar is poured on each to form a uniform thin film. Each slide is immediately replaced in the Petri dish and the agar allowed to solidify. The exudate which contains many streptococci in the bodies of the leucocytes is now gently smeared on these agar films, the watch glass is half filled with sterile water, and the large dish is placed in the incubator for four to six hours. The water is used to prevent drying of the agar.

Staining.—The slides are now taken out of the Petri dish and dried

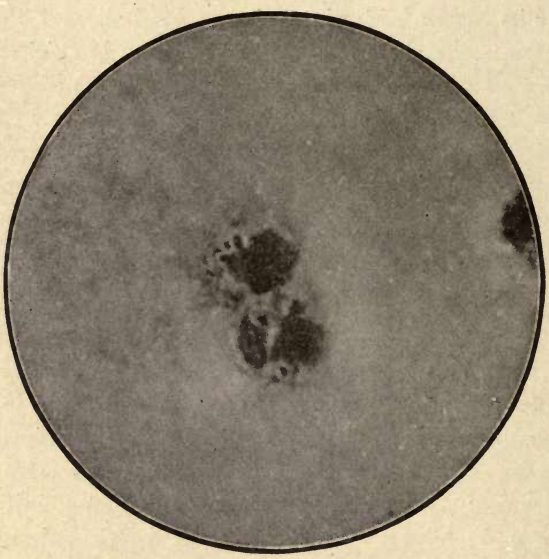

Fig. 1.—Photomicrograph × 1500. Streptococci in guinea-pig's leucocytes, taken from the pleura two hours after injection of streptococci.

in a thermostat at 55 C. for fifteen to twenty minutes, or longer if necessary; fixed in 40 per cent. formalin for four or five minutes, washed in water, stained for only a few seconds with Loeffler's methylene blue solution, again washed in water and dried in the thermostat.

The best stains were obtained when the methylene blue solution was poured over the wet slide, the slide being held so that the stain ran off readily. The slide is then immediately placed in a dish of cold water and gently rinsed in several changes of water. Violent agitation in the water pulls off the film of agar. When the agar is perfectly dry (which requires only about half an hour), a cover-glass is placed on it, either

in glycerin or Canada balsam. The slides are now ready to be examined with the oil immersion lens.

Examination.—Examination in various stages shows that the streptococci in the leucocytes multiply, burst open the cell and form a small colony surrounding the cell which contained them. Figures 2 and 3 are photomicrographs of such preparations at different stages. In Figure 2 the leucocyte has just been burst open and the streptococci are beginning to grow out of it; in Figure 3 a considerable colony has already formed around the ruptured leucocyte. These colonies may be found in all stages, by varying the time of incubation.

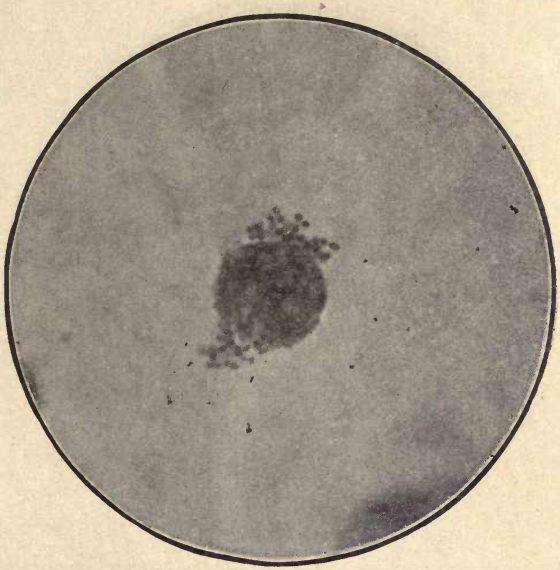

Fig. 2.—Photomicrograph ×1500. Multiplication of streptococci after their ingestion (*in vivo*) by a guinea-pig's leucocyte.

The leucocytes do, however, kill streptococci if the conditions are right, as is shown by the following two facts: 1. Organism No. 300 is not virulent for guinea-pigs, and we have seen that the leucocytes take up the cocci alive. The guinea-pig must destroy these organisms somewhere, and we can find no substance in its body that is capable of doing this except the leucocytes and the bone marrow. We have seen in Table 2 that the leucocytes destroy streptococci in vitro, and the inference is that they also destroy them in vivo. 2. Plates made with three loopfuls of the exudate when taken from the guinea-pig contained 2,540 colonies of streptococci after twenty-four hours' incubation, while plates made

with three loopfuls, after the exudate had stood in a test tube in the incubator for six hours, contained only 480 colonies. Evidently some of the streptococci were killed in this tube, but the smears show that all of the organisms are in the leucocytes, hence they must have been killed in the leucocytes.

EXPERIMENTS WITH LEUCOCYTIC EXUDATE IN VIVO.

In order to determine whether a rabbit is more resistant when injected in a pleura containing leucocytic exudate than when injected in the normal pleura, the following experiments were carried out:

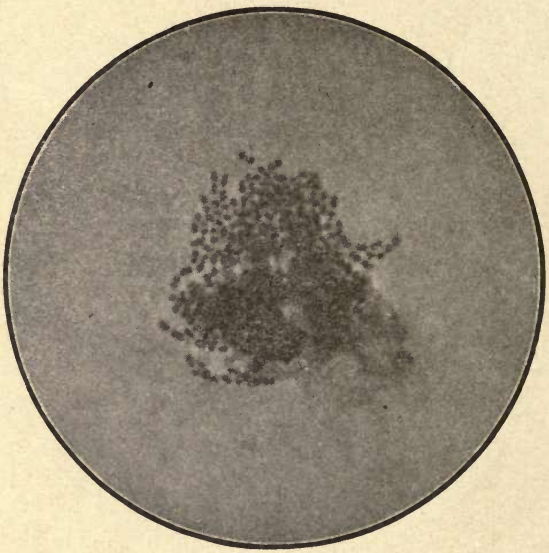

Fig. 3.—Photomicrograph × 1500. Multiplication of streptococci after their ingestion (*in vivo*) by a guinea-pig's leucocyte, in slightly later stage than Figure 2.

Experiment A.—A gray rabbit weighing 1,450 grams was injected into the right pleura with 7 c. c. of aleuronat suspension. Fifteen hours later 2 c. c. of a 40-hour culture of a virulent streptococcus were injected into this pleura and also into the right pleura of a normal rabbit weighing 1,500 grams. The normal rabbit was found dead in 18 hours, and the one which had been previously injected with aleuronat died 45 hours later.

Experiment B.—A gray rabbit weighing 2,100 grams was injected in the right pleura with 6 c. c. of aleuronat suspension. Eighteen hours later 1 c. c. of a 24-hour culture of a virulent streptococcus was injected

into the pleura and also into the right pleura of a normal gray rabbit weighing 2,150 grams. The normal rabbit died in about 18 hours, while the aleuronat rabbit died only after 45 hours—21 hours after the death of the control.

The experiment was now varied so that the conditions were the same as in Simon's experiments.

Experiment C.—A rabbit weighing 1,700 grams was injected in the right pleura with 8 c. c. of aleuronat suspension and 48 hours later this rabbit and a normal rabbit weighing 1,700 grams were each injected in the right pleura with 1 c. c. of a 30-hour streptococcus culture. In this experiment the rabbit treated with aleuronat died in 24 hours, while the control lived for five days.

Experiment D.—A rabbit weighing 1,500 grams was injected in the right pleura with 9 c. c. of aleuronat suspension. Forty-eight hours later this rabbit and a normal rabbit weighing 1,800 grams were each injected in the right pleura with 1.5 c. c. of a 24-hour streptococcus culture. The normal rabbit died in 37 hours, while the rabbit treated with aleuronat died in 45 hours—eight hours after the death of the control.

The results of the last two experiments agree fairly well with those obtained by Simon, and we may now ask why they are different from the results of Experiments A and B. Apparently this difference is due to the fact that in Experiments C and D the inoculation was made some thirty hours later than in Experiments A and B.

That this is the correct explanation is indicated by the following experiment:

Experiment E.—A rabbit weighing 2,300 grams was injected in the right pleura with 9 c. c. of aleuronat suspension. Forty-eight hours after this injection the rabbit was bled to death, and on opening the pleural cavity it was found to contain no leucocytic exudate.

Had this rabbit been inoculated in the right pleura forty-eight hours after the injection of the aleuronat suspension, the inoculation would not have been made into leucocytic exudate. That being the case we would not expect that this rabbit could stand a larger dose of streptococcus than a normal rabbit. In Simon's experiments, however, the inoculations into the pleural cavity were made forty-eight hours after the injection of aleuronat, and this is probably the reason why he did not find these rabbits more resistant than normal rabbits. Had he made his inoculations within twenty-four hours after the injection of aleuronat his results would very probably have been different.

At the suggestion of Professor Hektoen I tested filtrates of virulent streptococci to determine whether they are toxic for leucocytes, as has

been shown by Van de Velde,* and by Neisser and Wechsberg,** to be the case with staphylococcus filtrates. The organism used in these experiments was B104, which has been passed through 78 rabbits, and is very virulent for rabbits and for guinea-pigs, and produces an active hemolysin when grown in heated rabbit serum or in a mixture of equal parts of rabbit serum and ascites fluid. The filtrates used were from twenty-four to thirty-six-hour cultures in a mixture of rabbit serum and ascites fluid, which has been heated to 56C. for one-half hour before inoculation.

Neisser and Wechsberg have shown that living leucocytes reduce methylene blue solution when the supply of oxygen is shut off, while dead leucocytes no longer possess this property. In my experiments this fact was made use of to determine whether or not the leucocytes had been killed by the filtrate. 0.3 c. c. to 0.4 c. c. of leucocytic exudate from a rabbit was added to 1.5 c. c. of the filtrate, incubated for two hours, then two drops of methylene blue solution added and the contents of the tube shaken up and covered with a layer of sterile olive oil. If there was reduction in two hours the result was considered negative, but if there was no reduction in that time this fact was taken as evidence that the leucocytes were dead. Control tubes with a mixture of equal parts of heated rabbit serum and ascites fluid were always made, and the results recorded only when there was marked reduction in the control in two hours. A large number of these experiments were carried out, but it is not necessary to tabulate more than one of them here.

Experiment.—.3 c. c. exudate + 1.5 c. c. 24-hour filtrate; no reduction in 2 hours, but a trace of reduction in 3 hours.

.3 c. c. exudate + 1.5 c. c. 36-hour filtrate; no reduction in 3 hours.

.3 c. c. exudate + 1.5 c. c ascites-serum;* nearly complete reduction in 2 hours.

The leucocytic exudate was obtained from the pleura of a rabbit which had been previously injected with aleuronat suspension The methylene blue solution was made according to the formula given by Neisser and Wechsberg, which is given below:

Solution A.—Methylene blue, 1; absolute alcohol, 20; distilled water, 29.

Solution B.—1 c. c. of solution A; 49 c. c. of physiologic salt solution.

Two drops of solution B, which must be sterile, are added to each tube in the experiments.

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*An. de l'Inst. Pasteur, 1896, X, 580.

**Zeitschr. f. Hyg., 1901, XXXVI, 299.

*The term "ascites-serum" as used here refers to a mixture of equal parts of ascites fluid and rabbit serum, heated to 55 C. for one-half hour.

The fact that the filtrates have a decided effect on leucocytes has also been shown in a second experiment.

Experiment.—Two cubic centimeters of a rich leucocyitc exudate were placed in each of five small test tubes and centrifugated. The clear, supernatant fluid was now decanted from each tube and replaced by 2 c. c. of bouillon, heated ascites-serum or filtrate from a 24-hour ascites-serum streptococcus culture. The leucocytes in each tube were evenly suspended in the fluid, the tubes placed in a water bath at 37 C. and kept there for 1 to 1½ hours. Now .3 c. c. or normal rabbit serum (guinea-pig serum when guinea-pig's leucocytes were used) was added to each tube, the tubes inoculated with a loopful of a non-virulent streptococcus broth culture and plates made at intervals, the tubes being kept in the incubator.

The following table shows the results of an experiment:

	Immed.	4-5 hrs.	20 hrs.
Leucocytes + 2 c. c. filtrate B104 + .3 c. c. normal serum	2000	3200	Many
Leucocytes + 2 c. c. filtrate 10 A + .3 c. c. normal serum	1200	2000	Many
Leucocytes + 2 c. c. filtrate 300 + .3 c. c. normal serum	1100	24	Many
Leucocytes + 2 c. c. ascites serum + .3 c. c. normal serum	1000	140	Many
Leucocytes + 2 c. c. bouillon + .3 c. c. normal serum	1800	60	Many

The numbers B104, 10A and 300 refer to different strains of streptococci. B104 has a high virulence and 10A a medium virulence; both these strains produce hemolysin in heated serum, although 10A has never been passed through animals. It was recently isolated from suppurating axillary glands. 300 is non-virulent, and does not produce hemolysin.

The suspensions of leucocytes in filtrates from serum cultures of virulent streptococci have no effect on streptococci while the suspension in filtrates from non-virulent organisms, in heated ascites-serum, and in bouillon destroy large numbers of cocci.

These experiments do not always give positive results, but there is a small percentage of tubes in which the filtrate has apparently no effect on the leucocytes. This is not surprising, however, when we consider the fact that in about 100 filtrates which I have tested for hemolysis, there were nearly 10 per cent. that had no hemolytic properties, whereas some of them were so active that .005 c. c. completely laked 1 c. c. of a 2½ per cent. suspension of rabbit's washed corpuscles in two hours.

TEST TUBE EXPERIMENTS WITH HUMAN LEUCOCYTES.

The foregoing experiments show that the leucocytes and opsonin are the most important, if not the only, factors concerned in the destruction of streptococci in the body of infected rabbits and guinea-pigs. In strepto-

coccus infections in man the tissues are invaded by virulent streptococci but in a majority of these cases the cocci disappear sooner or later and the patients make a complete recovery. Freshly drawn human serum has no streptococcidal powers in vitro and it is reasonable to suppose that it has none in the body during life. We must look therefore for some other agent than the serum alone to account for the disappearance of the cocci from the tissues during convalescence. According to Metchnikoff and his followers this agent is found in the phagocytes and this view seems to explain the facts better than any other theory that has been advanced.

I next undertook a series of experiments to determine more accurately what factors are concerned in the destruction of the invading cocci in cases of human streptococcus infections, and to analyze more fully this phenomenon. In all instances where blood was used it was drawn from the vein at the elbow by means of a Luer syringe and defibrinated by gently whipping with a sterile wire. If the defibrinating is carefully performed not a very large proportion of the leucocytes are destroyed. In the experiments each tube contained from 0.8 to 1.0 c. c. of blood or serum which was inoculated with one loopful of streptococcus culture and two loopfuls from each tube were plated in glucose agar at intervals. The tubes were always kept in the incubator at 36 C. When highly virulent organisms were used 0.3 to 0.4 c. c. of defibrinated rabbit

TABLE 6.

THE EFFECT OF DEFIBRINATED NORMAL HUMAN BLOOD AND HUMAN SERUM ON STREPTOCOCCI.

Strepto-cocci.	Defibrinated Blood.	Colonies in Agar Plates. Immed.	2 to 3 hours.	5 hours.
300	I	1100	....	160
300	II	3000	....	1060
300	IIa	680	675	1300
300	III	2600	2700	3500
300	IV	3000	1050	2600
300	V	540	360	240
300	VI	1100	500	390
298	VII	1600	600	315
298	VII	1600	600	315
298	VIII	600	80	16
B104	IIa	76	55	510
B104	III	1800	2800	Many
B104	IV	1600	3000	Many
B104	V	500	600	900
381	VI	2000	2300	Many
300	Serum IV	1400	2000	Many
298	Serum VII	1700	2500	10000

blood was added to each tube of melted agar to facilitate the counting of colonies, which often are very small if no blood has been added.

Table 6 shows that, although human serum in vitro is a good culture medium for streptococci, normal defibrinated blood has a slight streptococcidal power. Occasionally we may find a sample of normal blood which destroys many non-virulent streptococci, but the virulent organisms usually multiply in this blood. Table 7 shows that defibrinated blood from patients suffering from an acute infection has a much greater destructive effect on these organisms than has normal blood. (The non-virulent cocci are ingested very freely by the leucocytes while the virulent strain is not taken up so freely, as shown by counts in stained smears.)

TABLE 7.

THE EFFECT OF DEFIBRINATED BLOOD FROM CASES OF SCARLATINA, ERYSIPELAS AND PNEUMONIA ON STREPTOCOCCI.

Strepto-cocci.		Defibrinated Blood.	Leucocyte count.	Colonies in Agar Plates. Immed.	2 to 3 hours.	5 hours.
300	Scarlatina	I	10500	1100	....	6
300	"	II	10600	2000	....	140
300	"	III	15000	690	270	116
300	"	IV	.....	550	18	3
300	"	V	.....	1500	300	150
300	"	VI	.....	1900	350	43
300	"	VII	.....	500	32	12
300	"	VIII	13000	1380	102	9
300	"	IV	.....	1200	125	14
300	"	X	13400	1250	245	120
300	"	XI	.....	1950	150	19
300	"	XII	10800	1800	700	130
300	"	XIII	.....	1800	300	45
300	"	XIV	12300	1500	31	2
381	"	III	15000	1200	1500	3500
B104	"	IV	.....	600	462	415
B104	"	V	.....	1800	1800	5000
381	"	VI	.....	220	51	1100
381	"	VII	.....	130	41	Many
381	"	VIII	13000	360	1180	Many
381	"	IX	13000	2200	1950	Many
B104	"	IX	.....	330	190	1500
381	"	X	13400	640	290	2500
B104	"	X	.....	400	160	165
381	"	X	.....	320	650	Many
381	"	XII	10800	200	2000	Many
381	"	XIII	.....	540	900	Many

300	Erysipelas	I	.....	630	64	0
300	"	II	15000	800	14	4
300	"	IIa	.....	1100	41	8
300	"	III	.....	740	120	160
300	"	IV	.....	1650	480	150
298	"	V	10400	3100	420	92
B104	"	I	.....	1200	1080	960
B104	"	II	.....	96	30	13
B104	"	IV	.....	133	110	480
300	Tonsillitis	I	16000	620	34	2
B104	"	I	.....	300	59	70
300	"	II	.....	1400	74	6
300	Pneumonia	1	.....	2600	0	3
300	"	II	.....	600	5	0
	Serum					
300	Scarlatina	VII	.....	1500	3500	10000
300	"	XIV	.....	1200	1400	1300
300	Erysipelas	II	.....	670	760	750
300	"	III	.....	700	1800	6000
300	"	V	.....	3000	3500	5000
B104	"	V	.....	190	360	1140

In these infections the leucocyte count is usually somewhat increased, and it seems that the streptococcidal power of the blood in vitro is roughly proportional to the leucocyte count. That is, the higher the leucocyte count the greater will be the streptococcidal power of the blood. This is a general rule, to which there are, however, a few exceptions, as will be pointed out later. The virulent organisms frequently multiply in these bloods unless the leucocytosis is very high. In no instance could a streptococcidal power of the serum alone be detected. It might be objected that we are not dealing here with an actual destruction of cocci, but that the decrease in the number of colonies on the plates is due to adherence of the cocci to the leucocytes. This objection is ruled out by the fact that the twenty-four-hour plates from tubes containing blood with a high leucocytosis are very often sterile or nearly so.

It has been thought possible that the serum during the course of an infection which terminates favorably might acquire streptococcidal properties for that particular race of streptococci which is responsible for the infection, while at the same time it had no such properties for other races of these organisms. Three strains of streptococcus were, therefore, isolated from erysipelas patients and the serum of each patient tested on the corresponding organism. All of the patients made a satisfactory recovery, but at no time could streptococcidal properties be demonstrated in their serum. The defibrinated blood, on the other hand,

killed many of the homologous and other organisms as long as there was a high leucocytosis.

We know that there is an intense local reaction in the localized streptococcus infections, and it has been thought by some that there may be lysis of cocci by the inflammatory serum in these areas. It is difficult to confirm or refute this theory on account of the difficulty of obtaining inflammatory serum in the same condition as it is found in the tissues. As it is not uncommon to find blebs of considerable size on the affected parts of erysipelas patients, the fluid from these blebs was taken as the nearest approximation to the inflammatory serum. This blister fluid from several cases of erysipelas was tested for streptococcidal properties but gave negative results.

The importance of a high leucocyte count in the destruction of streptococci by blood is clearly shown by the following experiment:

Experiment 1. Ten cubic centimeters of blood were drawn from the vein at the elbow of an erysipelas patient and carefully defibrinated. One cubic centimeter of the defibrinated blood, which contained 9,800 white corpuscles per cubic millimeter, was put into a small test tube, inoculated with one loopful of virulent streptococcus culture, and two loopfuls of the inoculated blood were plated at intervals. The remaining eight cubic centimeters were centrifugated and the serum drawn off. We know that the uppermost stratum of centrifugated corpuscles contains a high percentage of leucocytes, because they are thrown down less easily than the red corpuscles. This stratum was therefore drawn off with a sterile pipette and mixed with a small quantity of serum. The resultant mixture contained 17,200 leucocytes per cubic millimeter. One cubic centimeter of this "suspension of leucocytes" was introduced into a small test tube, inoculated and plates made as before. To complete the experiment one cubic centimeter of the clear serum was put into a small tube, which was likewise inoculated, and plates made at intervals. This experiment was also performed with normal blood and a non-virulent streptococcus. The plates were incubated for twenty-four hours,

TABLE 8.

Strepto-cocci.		Leucocyte count.	Colonies on Agar Plates. Immed.	2 to 3 hrs.	5 hrs.
B104	Erysipelas blood	9800	390	168	350
B104	Suspension erysipelas leuc.	17200	260	58	21
B104	Erysipelas serum	....	200	360	1100
298	Normal blood	4400	1650	600	315
298	Suspension normal leuc.	6600	1600	420	62
298	Normal serum	....	1700	2500	10000

and the colonies that developed on each were counted with the results shown in Table 8.

The table shows that both strains of streptococcus used multiplied in the cell-free serum; that the defibrinated blood destroyed many of the non-virulent and some of the virulent cocci, and the "suspension of leucocytes" destroyed more cocci of either strain than the defibrinated blood. The only difference between the defibrinated blood and the "suspension of the leucocytes" lay in the fact that the latter contained nearly twice as many leucocytes as the former.

The fact that the streptococcidal power of the blood is dependent on the number of leucocytes it contains per cubic millimeter has also been demonstrated by a second experiment.

Experiment 2.—Shortly after a patient's admission into the hospital, 3 c. c. of blood was drawn from a vein at the elbow and defibrinated. The leucocyte count at this time was 11,000. The blood was divided equally among three tubes, and each tube was inoculated with streptococcus culture and two loopfuls from each were plated at intervals. Shortly after drawing the blood, the patient was injected under the skin of the back with 10 c. c. of an antistreptococcus serum. This brought about an increase in the leucocytes up to 15,000, five hours after the injection. Three c. c. of blood was again drawn from the vein at the elbow and its effect on streptococci tested as before. The results of the experiments are shown in Table 9.

TABLE 9.

Streptococci.		Leucocyte count.	Colonies on Agar Plates. Immed.	2 to 3 hrs.	5 hrs.
300	Blood before injection	11000	1100	360	200
B104	Blood before injection	....	390	380	4000
381	Blood before injection	....	100	500	Many
300	Blood 5 hours after injection	15000	1300	44	0
B104	Blood 5 hours after injection	....	412	246	600
381	Blood 5 hours after injection	....	150	270	3000

The blood drawn after the injection of the serum, when the leucocytosis was high, has a greater streptococcidal power than that drawn before the injection. It would not be safe to conclude that this difference in the streptococcidal power is due entirely to the difference of the leucocyte count. Some of it may be due to an antitoxic or opsonic action of the serum or to a stimulation of the leucocytes. This supposition loses most of its force when we consider the fact that the addition of from 1 to 5 per cent of this antistreptococcus serum to defibrinated blood in vitro

does not increase its streptococcidal power, as shown by experiments by Hektoen & Ruediger.

We know that untreated bacteria are not taken up by washed leucocytes in Na Cl solution or in heated serum, and hence there should be no reduction in the number of streptococci in a test tube containing a suspension of washed human corpuscles in salt solution or in heated serum. The following experiment shows that this is the case:

Experiment 3.—Ten c. c. of blood was drawn from a vein at the elbow of a scarlet fever patient, defibrinated, centrifugated and the serum drawn off. The corpuscles were washed twice in a large amount of NaCl solution, and 0.5 c. c. of the washed corpuscles placed into each of three small tubes containing 0.5 c. c. of normal serum, 0.5 c. c. of heated serum (58 degrees for one-half hour) and 0.5 c. c. of salt solution, respectively. The tubes were inoculated with one loopful of streptococcus culture, and two loopfuls from each were plated at intervals with the results shown in Table 10.

TABLE 10.

Strepto-cocci.		Colonies on Glucose Agar Plates. Immed.	5 hours.	24 hours.
300	Washed corpuscles + serum	1500	7	21
300	Washed corpuscles + heated serum	1400	5000	Many
300	Washed corpuscles + NaCl solution	1700	3500	Many
300	Serum	1200	1400	Many

The importance of opsonin in the destruction of streptococci by human leucocytes is further shown by the fact that the defibrinated blood from two obstinate cases of post-scarlatinal nephritis had no streptococcidal powers, although in one of the cases the leucocyte count was 14,000. When the blood from these patients was centrifugated and the corpuscles suspended in serum from a patient who was convalescent and had no nephritis, the resultant suspension had a small degree of streptococcidal power, as shown by Table 11.

The interesting fact came to light in these experiments that the combination "convalescent washed corpuscles plus nephritis serum" has

TABLE 11.

Strepto-cocci.		Colonies on Agar Plates. Immed.	2 hours.	5 hours.
298	Convalescent blood	800	450	14
298	Nephritis blood	720	1900	6000
298	Nephritis corpuscles + conv. ser.	850	500	540
298	Conv. washed corp. + Neph. ser.	725	600	61

nearly as great a streptococcidal power as the defibrinated blood from the patients without nephritis. The corpuscles were washed twice in a large amount of NaCl solution which is usually sufficient to prevent phagocytosis in a suspension of corpuscles in NaCl solution. It is not likely, therefore, that the washing had not been carried far enough. But the results of these experiments seem to indicate rather that the leucocytes as well as the serum from these nephritis patients have undergone some change which renders them less efficient in the destruction of bacteria. In fact, it would seem that the leucocytes have suffered more than the serum. Whether or not these facts may serve to throw light on the cause of some of the terminal infections can not be determined at this time.

It is an interesting question whether the opsonin is increased or not during the acute infections. Normal leucocytes in normal serum take up large numbers of cocci; hence, it is difficult to determine if leucocytes in erysipelas serum, for instance, take up more cocci than those in normal serum. This question had, therefore, to be approached in a different way.

Experiment 4.—Two sets of tubes were made and 0.2 c. c. of washed corpuscles introduced into each. To one set of tubes were added falling quantities of normal serum and to the other set falling quantities of erysipelas serum. The contents of each tube were made up to 0.4 c. c. with NaCl solution, and to each tube was added 0.4 c. c. of a suspension of streptococci. The tubes were incubated for one hour at 36 C., smears were made and the average number of cocci in each leucocyte determined by counting those in 30 leucocytes. The results are shown in Table 12.

Table 12.

Normal serum.	Phagocytosis.	Erysipelas serum.	Phagocytosis.
0.2 c. c.	13.5	0.2 c. c.	11.5
0.1 c. c.	9.4	0.1 c. c.	11.2
0.05 c. c.	6.7	0.05 c. c.	8.2
0.025 c. c.	4.7	0.025 c. c.	6.9
0.012 c. c.	2.2	0.012 c. c.	4.3

This experiment indicates that there is a slight increase of opsonin in the erysipelas serum as compared with normal serum. A similar increase of opsonin has been noted by Wright and Douglas* after treating with their staphylococcus vaccine a person afflicted with furunculosis. Hektoen** also observed a rise in opsonin after injecting a person with

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**Journ. A. M. A., 1906, XLVI, 1409.

heated streptococci. A diminution of opsonin in persons subject to attacks of furunculosis, sycosis, etc., has also been observed by Wright and Douglas.

During the course of this work I had an opportunity to study a case of acute purulent rhinitis, in the discharge of which streptococci were found in pure culture.

Patient.—A man 27 years of age. During the first two days the discharge was clear and watery and contained very many streptococci in short chains and in diplococci. Figure 4 shows a photomicrograph of a smear make on the second day. There were only a few leucocytes, and those did not contain any streptococci. On the fourth day the discharge

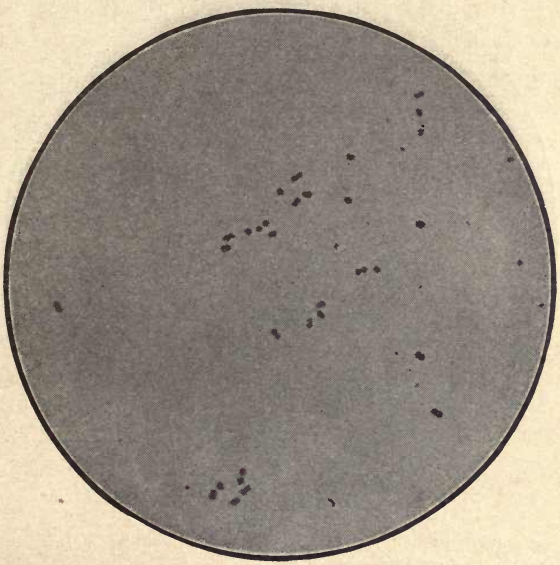

Fig. 4.—Photomicrograph × 1500. Streptococci in nasal discharge on second day of sickness.

had become thick and purulent, and smears made at that time presented an entirely different picture. There were no free streptococci, but very many leucocytes, some of which were loaded with streptococci. Figure 5 shows a photomicrograph of such a preparation made on the fourth day. The purulent discharge continued for about a week, and during this time it was easy to find leucocytes in the smears which contained large numbers of streptococci. The streptococci finally disappeared, and the patient made a complete recovery.

In this case of streptococcus infection phagocytosis of the organisms by the leucocytes seemed to be an important factor in combating

the infection. In the smears made on the fourth day no free streptococci could be found, although diligent search was made for them. Plates made with a small quantity of discharge, which was obtained high up in the nares by means of a sterile cotton swab, contained, however, many colonies of streptococci after twenty-four hours' incubation. The fact that many colonies developed in the plates, although no free streptococci could be found in the smears, seems to indicate that the organisms were not dead when they were taken up by the phagocytes, but were taken up alive. It is possible, however, that there were free streptococci in this purulent discharge, even though they were not found in the smears.

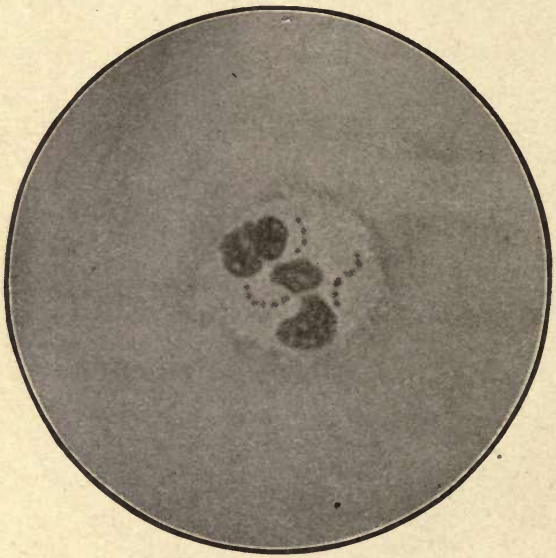

Fig. 5.—Photomicrograph × 1500. Streptococci in a leucocyte from nasal discharge on fourth day of sickness.

IMMUNIZATION OF RABBITS WITH STREPTOCOCCI.

There can no longer be any doubt about the statements of many investigators that laboratory animals acquire a fair degree of immunity after repeated injections of small doses of virulent streptococci. I have injected a large number of rabbits first with several doses of heated cultures (65° C. for 20 minutes) and then with living cultures of medium virulence, and have found that some animals acquire enough resistance after 4 or 5 injections to withstand injections of several times the minimum fatal dose of the streptococcus. Thus, for instance, a full grown rabbit weighing 2000 G. had received 2 large injections of heated culture

and 4 small injections of the living culture "381" at intervals of 8 to 10 days. This rabbit was now injected under the skin of the back with 4 c. c. of a suspension of streptococcus "381" and at the same time a normal rabbit weighing 2100 G. was injected in the corresponding locality with 2 c. c. of the same streptococcus suspension. The normal rabbit died on the 3rd day while the immune rabbit showed no signs of sickness except that it lost 150 G. in weight, which was regained at the end of one week.

Experiments with rabbit leucocytes in immune serum in vitro. Experiments were now undertaken to make a further study of the mechanism of this immunity than has already been made by Neufeld and Rimpau and others.

An immune rabbit and a normal rabbit were each bled 5 c. c., the bloods defibrinated, divided into two equal quantities and leucocytes added to each blood. One portion of each blood received leucocytes from the normal rabbit and the other portion received washed leucocytes from the immune rabbit. The whole blood instead of the serum alone was used because rabbit blood contains only a very small number of polymorphonuclear leucocytes which may be neglected, and it has been found that smears from tubes containing leucocytes in blood are more

TABLE 13.

PHAGOCYTOSIS OF STREPTOCOCCI BY NORMAL AND BY IMMUNE RABBIT LEUCOCYTES IN NORMAL AND IN IMMUNE RABBIT BLOOD.

Strepto-cocci.	Phagocytosis by normal leucocytes in: Normal Blood.	Immune Blood.
Laura*	6.2	14.
381	2.2	4.8
Joe R.	4.4	7.6
Stuart	1.	4.5
Puerperal	1.	1.6
	Phagocytosis by normal leucocytes in: Normal Blood.	Immune Blood.
Laura	5.5	15.2
381	1.7	5.
Joe R.	5.3	7.2
Stuart	1.	4.2
Puerperal	5.	2.5

**Sources of Streptococci: "Laura" from a scarlatinal otitis media, and used to immunize the rabbit, the serum of which was used in this experiment; "Joe R." from a scarlatinal sore throat; "381" from pericardial fluid of a scarlatinal body; "Stuart" from a case of erysipelas and "Puerperal" from a blood culture of a case of puerperal sepsis. All were only of medium virulence.

satisfactory than those made from suspensions of leucocytes in serum. Four-tenths of a cubic centimeter of each suspension of leucocytes in blood was put into a number of small test tubes and .2 c. c. of a fairly thick suspension of virulent streptococci added to each tube. The tubes were incubated at 36 C. for 1 hour, smears were made and the average number of cocci ingested by each leucocyte was determined by counting the cocci in 30 leucocytes. The results of such an experiment are shown in Table 13.

The Table shows that there is a larger number of cocci taken up by the leucocytes in immune blood than by those in normal blood; that the immune leucocytes do not ingest appreciably more cocci than the normal leucocytes and that the increase is most marked with that strain of streptococcus which was used in immunizing the rabbit.

Four other experiments with the bloods of different immune rabbits gave practically the same results. The increased phagocytosis always was most pronounced with that strain of streptococcus which had been used in immunizing the rabbit whose defibrinated blood or serum was used in the experiment. Several strains of streptococcus were used in the immunization and all that were of medium virulence gave an immunity that could be demonstrated in the test tube. Rabbits were also treated with a highly virulent streptococcus, but this organism was scarcely taken up at all by rabbit leucocytes suspended in this or in other immune sera.

An experiment was now carried out to show how the immune serum promoted phagocytosis of the virulent cocci. For this purpose normal rabbit leucocytes were suspended in normal and some in immune rabbit serum for ½ hour, then centrifugated out of the sera, washed twice in NaCl solution and suspended in normal serum. To each tube was now added the same amount of suspension of a virulent streptococcus, the tubes were incubated for 1 hour, smears were made from each and the degree of phagocytosis determined by counts. In neither tube was there any phagocytosis, showing that the immune serum is not capable of changing the leucocytes so that they will ingest virulent streptococci which have not been treated with immune serum. A second pair of tubes was carried through at the same time, using the same leucocytes, streptococcus suspension and sera. In this instance virulent streptococci were treated ½ hour with immune rabbit serum and some with normal rabbit serum, then washed once in a large amount of NaCl solution, suspended in NaCl solution and each lot added separately to a suspension of normal rabbit leucocytes in NaCl solution. The tubes were incubated 1 hour, smears were made as above and the degree of phagocytosis determined by counts. In this pair of tubes the degree of phago-

cytosis was strikingly different. The cocci which had been sensitized in normal serum were not ingested by the leucocytes, while those that had been sensitized in immune serum were taken up freely. That is, the serum has acquired something in the process of immunization by virtue of which it is capable of so changing the virulent streptococci that they become susceptible to phagocytosis. The results of the count are shown in Table 14.

TABLE 14.

PHAGOCYTOSIS OF VIRULENT STREPTOCOCCI SENSITIZED IN IMMUNE RABBIT SERUM.

1.	Washed rabbit leucocytes + normal rabbit serum + streptococcus "Laura"	0.4
2.	Rabbit leucocytes suspended in immune rabbit serum ½ hour, washed in NaCl solution, resuspended in normal serum + streptococcus "Laura"	0.6
3.	Washed rabbit leucocytes in NaCl solution + streptococcus "Laura" sensitized in normal rabbit serum	0.6
4.	Washed rabbit leucocytes in NaCl solution + streptococcus "Laura" sensitized in immune rabbit serum	8.0

The destruction of virulent streptococci by rabbit leucocytes in immune serum can also be shown by means of plate cultures. For this purpose 2 tubes were prepared containing (a) 1 c. c. defibrinated normal blood + .2 c. c. of a thick suspension of rabbit leucocytes, and (b) 1 c. c. defibrinated immune blood + .2 c. c. of the same suspension of leucocytes. Each tube was inoculated with one loopful of a virulent streptococcus culture, incubated at 36° C. and plates made at intervals with 2 loopfuls of blood from each tube. The plates were incubated for 24 hours and the colonies carefully estimated with the following result:

Strepto-cocci.		Colonies in Agar plates. Immed.	2 hours.	5 hours.
381	Leucocytes in normal blood	480	1200	8000
381	Leucocytes in immune blood	460	300	5000

In the tube containing immune blood + leucocytes the number of organisms decreased during the first two hours but then began to increase rapidly, while in the other tube they increased rapidly from the beginning of incubation. The immune serum alone has no destructive action on these organisms but acts as a culture medium for them.

The following experiment was now made to determine whether the opsonin of the immune serum can withstand a higher degree of heat than that of normal serum.

Experiment.—.2 c. c. of normal serum and .2 c. c. of immune serum in two small test tubes were heated to 60° C. for ½ hour. To each

tube were now added .2 c. c. suspension of washed rabbit corpuscles containing leucocytes from a pleural exudate and .2 c. c. suspension of streptococci of medium virulence. Two additional tubes with unheated normal and immune serum were prepared in the same manner and all tubes incubated ½ hour. Smears were now made from all tubes and stained and the degree of phagocytosis in each tube determined by counting the cocci in each of 50 leucocytes, with the following result:

	Phagocytosis.
Heated normal serum + washed corpuscles + streptococci	1.1
Heated immune serum + washed corpuscles + streptococci	4.5
Normal serum + washed corpuscles + streptococci	4.3
Immune serum + washed corpuscles + streptococci	7.3
Washed corpuscles in NaCl solution + streptococci	1.1

The experiment shows that the opsonin of the immune serum is only slightly disturbed by heating at 60 C. for ½ hour, whereas that of normal serum is entirely destroyed at that temperature.

CONCLUSIONS.

The normal sera of man, rabbits and guinea-pigs have no streptococcidal power and do not acquire such a property in the course of a streptococcus infection.

Suspensions of organ cells of guinea-pigs have no streptococcidal powers.

Defibrinated human blood is distinctly streptococcidal and this property is roughly proportional to the number of leucocytes the blood contains per cubic millimeter.

Normal leucocytes of rabbit, guinea-pig and man, suspended in normal serum or blood, freely ingest non-virulent streptococci and destroy them. The washed leucocytes in NaCl solution or in heated serum do not ingest these organisms, but the latter multiply in these suspensions. If, however, the streptococci are treated with normal serum, washed and then added to a suspension of washed leucocytes they are ingested and destroyed by the leucocytes. In the phagocytosis of streptococci, therefore, it is essential that the organisms should first be sensitized, that is, acted upon by the opsonin of the serum.

The leucocytes of guinea-pigs and of man take up living streptococci in vivo, and in all probability destroy them. It seems evident, therefore, that the phagocytes, acting in conjunction with the opsonin of the serum, are the most important (if not the only) factors concerned

in combating streptococcus infections in man and in the lower animals.

Hektoen and Ruediger* have shown that m-8 solutions of many salts and other substances inhibit phagocytosis, presumably because these substances bind the opsonin. I have carried out a number of experiments with rabbits for the purpose of determining whether or not intravenous injections of weak solutions of antiseptics can prolong the animal's life when inoculated with a fatal dose of streptococcus, but have found that the animals thus treated invariably died earlier than the controls. We must conclude, therefore, that great harm can be done by the indiscriminate use of drugs, or antiseptics, for the purpose of combating streptococcus infections. The remedy used may act on the opsonin, for instance, so as to hinder phagocytosis and thus do harm rather than good.

Virulent streptococci are not freely, or scarcely at all, ingested by normal leucocytes suspended in normal serum or blood, and hence these organisms multiply in suspensions of leucocytes. Human leucocytes take up virulent streptococci somewhat more freely than do the leucocytes of rabbits and guinea-pigs.

Rabbits can be successfully immunized with streptococci of medium virulence, and this immunity is clearly dependent upon phagocytosis. The immune rabbit serum has no streptococcidal power, but the leucocytes suspended in the immune blood or serum readily take up and destroy that strain of streptococcus which was used in the immunization. Other strains of virulent streptococci are not taken up so readily by leucocytes suspended in the immune serum. This is an important fact to be recognized in the serum therapy of streptococcus infections.

The action of the immune serum is not that of a stimulus for the leucocytes but the effect is on the cocci. The immune serum has acquired the power to change the cocci so that the leucocytes will ingest them, this power being possessed only slightly by the normal serum.

The opsonin of human serum is increased during the course of a streptococcus infection, and according to Hektoen also after a subcutaneous injection of heated streptococci.

The immune opsonin is more resistant to heat than normal opsonin.

*Jour. of Infect. Dis., 1905, II, 128.